The hydrolysis of phosphorylcholine-containing metabolites in plant tissues: partial purification of a CDP-choline hydrolase from *Solanum tuberosum**

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Five plant tissues were extracted and found to contain enzymes which could hydrolyze *p*-nitrophenylphorylcholine (PNP-PC), an artificial substrate which has been reported to be a convenient substrate for measuring the activity of phospholipase C. The leaves and tubers of *Solanum tuberosum* L. were then examined in more detail and found to contain enzymes which were capable of hydrolyzing several phosphorylcholine-containing metabolites. These metabolites included phosphatidylcholine (PC), glycerol phosphorylcholine, and cytodine diphosphocholine. The PNP-PC hydrolase from potato leaves was partially purified (a 229-fold increase in specific activity was achieved) and was found to have a molecular weight of about 28,000 and an isoelectric pH of approximately 6.8. When PNP-PC was used as a substrate, the enzyme was found to exhibit optimal activity at pH 6.0, was inhibited by Zn^{2+}, and was found to have a K_m of about 10 mM for PNP-PC. This hydrolytic enzyme activity was then tested for activity with naturally-occurring phosphorylcholine-containing metabolites and was found to hydrolyze CDP-choline but not PC or glycerol phosphorylcholine. This report indicates that although several plants contain enzymes that are capable of hydrolyzing PNP-PC, this substrate appears to be hydrolyzed by enzymes other than C-type phospholipases.

Introduction

Many types of plant tissue have been shown to contain high levels of enzymes which degrade phospholipids. The two most common types of phospholipases in plants are phospholipase D and lipolytic acyl hydrolase (which hydrolyzes the fatty acyl esters found in both phospholipids and galactolipids). Recently, one group reported the occurrence of a phosphatidylinositol-specific phospholipase C in plants [1]. There have been five other reports of nonspecific phospholipase C activities in plants [2—6]. In three of these studies [4—6], the activity of phospholipase C was measured using an artificial substrate, *p*-nitrophenylphosphorylcholine (PNP-PC), which had previously been reported to be useful as a substrate for C-type phospholipases [7]. This study was undertaken to investigate the occurrence of phospholipase C in potato leaves and tubers and to evaluate the usefulness of PNP-PC as a substrate for phospholipase C, or any other hydrolytic activities (for example, hydrolysis of CDP-choline or glycerol phosphorylcholine).

Materials and Methods

Certified virus-free seed potato tubers (*Solanum tuberosum* cv. Kennebec) were obtained from Agway. Plants were grown from tubers as previously described [8]. Broccoli and cauliflower were obtained from a local market. PNP-PC and the other

phosphorylcholine-containing metabolites and lipids were obtained from Sigma Chemical Co. [*methyl*-^{14}C]CDP choline was obtained from New England Nuclear.

Homogenization and preparation of supernatant fraction

Leaves (50 g of leaves, each 2 to 8 cm in length) were picked and homogenized with a chilled mortar and pestle in homogenization medium (100 ml) which contained 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.5), 5 mM dithiothreitol (DTT) and 5 mM β-mercaptoethanol. When tubers or florets were studied they were peeled and diced into small pieces (1 cm^2) and homogenized in the same buffer for 60 s at high speed in a Waring Blendor. The homogenates were filtered through 2 layers of cheesecloth and centrifuged at 100 000 $\times$ *g* for 50 min. All operations were performed at 0—4°C.

Enzyme purification

The 100 000 $\times$ *g* supernatant fraction was first applied to a column of Sephadex G-25 (2.5 $\times$ 50 cm) and eluted with 0.1 M HEPES-NaOH (pH 7.0). All of the PNP-PC hydrolase activity was eluted in the void volume. The enzyme activity passed through a DEAE cellulose column (2.0 $\times$ 10 cm) when rinsed with 0.1 M HEPES-NaOH (pH 7.0). The rest of the protein was removed from the DEAE column with 0.5 M NaCl. The active fraction from the DEAE column was then applied to a Red A (Reactive Red 120-Agarose) column (1 $\times$ 10 cm). The column was rinsed with 15 ml of 0.02 M HEPES-NaOH (pH 7.0), followed by a gradient of 0—0.1 M KCl in the same buffer. The peak of activity from the Red A column was pooled, concentrated by ultrafiltration with an Amicon apparatus (equipped with PM-10 membranes) and applied to an LKB Ultrogel AcA-34 gel filtration column (1.0 $\times$ 50 cm) which was eluted with 25 mM imidazole buffer (pH 7.4). The peak of activity from the AcA column was then pooled and applied to a chromatofocus column (Pharmacia Polybuffer Exchanger 94, 0.8 $\times$ 8.0 cm) which was preequilibrated with 25 mM imidazole buffer (pH 7.4). The column was then eluted with diluted (1:8) Pharmacia Polybuffer 74 (adjusted to pH 4.0 with hydrochloric acid) to produce a linear pH gradient.

Electrophoresis

During the enzyme purification, each stage of purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini-Protean II and the conditions of Laemmli [9]. Gels were stained either with Coomassie blue R-250 or with silver stain according to the method of Merril et al. [10].

Enzyme assays

The hydrolysis of PNP-PC was measured by a modification of the method of Kurioka and Matsuda [7]. Sorbitol was deleted because initial studies revealed that it had no effect on enzyme activity. The reaction mixture (1 ml) contained 1 mM PNP-PC, 0.05 M Tris—HCl buffer (pH 7.0) and 5—100 μl of enzyme.

The hydrolysis of phosphatidylcholine (PC, dioleoyl), phosphatidylethanolamine and phosphatidylinositol, was measured by dispersing each substrate (10 mM) in water or 0.1% Triton X-100 for 5 min in a bath Ultrasonic Generator (Model G 1225P IT, Laboratory Supplies Col, Hicksville, NY). The stock lipid emulsions were then added to buffer (to give a final concentration of 1.0 mM) and incubated with various test solutions of enzyme. After incubation, the reactions were stopped by adding 100 μl of acetic acid and the lipids were extracted with hexane-isopropanol [8]. The rate of release of diacylglycerols, phosphatidic acid, fatty acids, or other potential lipid products was measured by a new high performance liquid chromatography (HPLC) technique which utilizes a flame ionization detector to quantify the mass of each lipid class [8].

The hydrolysis of ^{14}C-labelled CDP-choline or glycerol phosphorylcholine (unlabelled) was measured in a reaction mixture containing either potential substrate at 1 mM, 10 mM cacodylate buffer (pH 6.0) and 100—400 μl of enzyme sample. Samples were incubated for designated periods of time at 25°C and the reactions were stopped by freezing the tubes in liquid nitrogen. The frozen samples were then lyophilized, redissolved in 50 μl of methanol/water (1:1) and the entire sample was spotted onto a thin layer chromatography (TLC) plate (silica gel G). Standards of choline, phosphorylcholine, glycerol phosphorylcholine, and CDP-choline were also spotted on the TLC plate,

and it was developed in a mixture of 60:30 (v/v) 7.4 M ammonium hydroxide:methanol. The plate was then air dried for 30 min and placed in an I_2 tank to visualize the various metabolites. In this TLC technique the standards had the following R_f values: choline (0.89), glycerol phosphorylcholine (0.78), CDP-choline (0.70) and phosphorylcholine (0.56). The lanes that corresponded to samples which had contained [^{14}C]CDP-choline were scanned for radioactivity with a Berthold Model 282 Linear Analyzer. When glycerol phosphorylcholine was tested as a potential substrate, the spots which cochromatographed with authentic phosphorylcholine were then scraped with a razor blade and the level of phosphorus was determined by using a spectrophotometric assay [8]. The amount of phosphorus was then used to calculate the corresponding level of phosphorylcholine produced in each sample.

Estimation of molecular weight

The molecular weight of the partially purified PNP-PC hydrolase was estimated by placing the enzyme preparation on a second gel filtration column (LKB Ultrogel AcA-34, 1 × 50 cm, eluted with 50 mM HEPES-NaOH, pH 7.0). Four protein molecular weight standards and blue dextran were used to calibrate the column.

Results

Hydrolysis of PNP-PC by various plant tissues

In the first experiment (Table I) supernatant fractions (100 000 × g) were prepared from five plant tissues and each was found to be able to hydrolyze PNP-PC, a compound which had previously been proposed as a convenient substrate for phospholipase C activities [7]. The PNP-PC hydrolase activities ranged from 5.9 to 72.8 nmol min^{-1} ml^{-1}, with potato leaves exhibiting the highest level of activity (per ml of supernatant fraction) and broccoli florets exhibiting the lowest. When expressed in terms of specific activity the values ranged from 2.0 to 8.5 nmol min^{-1} (mg prot.)$^{-1}$.

Ability of crude plant supernatant fractions to hydrolyze various substrates

Supernatant preparations from potato leaves and tubers were then tested for their ability to hydrolyze PNP-PC and three physiological phosphorylcholine-containing compounds (Table II). Leaves and tubers were found to contain enzymes capable of hydrolyzing all four compounds. Using a TLC assay technique the product of hydrolysis of CDP-choline in the crude enzyme preparations was found to be phosphorylcholine (and presumably CMP). Using the same TLC assay technique the product of hydrolysis of glycerol phosphorylcholine was found to be phosphorylcholine (and presumably glycerol).

Table I. A comparison of the rates of hydrolysis of PNP-PC by supernatant preparations from several plant tissues.

Plant tissue	PNP-PC hydrolase	
	nmol min^{-1} ml^{-1}	nmol min^{-1} (mg prot.)$^{-1}$
Potato leaves	72.8	4.6
Potato tubers	17.0	8.5
Tomato leaves	38.3	7.2
Cauliflower florets	14.5	5.3
Broccoli florets	5.9	2.0

Purification of PNP-PC hydrolase

Because potato leaves exhibited a high rate of PNP-PC hydrolysis (Table I), we chose this plant tissue to purify the activity and study its properties (Table III). The first step of purification consisted of placing the 100 000 *g* supernatant on a Sephadex G-25 column to remove the pigments, phenolics, and other low molecular weight materials. The G-25

Table II. Comparison of the rates of hydrolysis of various phosphorylcholine-containing metabolites by crude supernatant preparations from potato leaves and tubers. All assays were buffered at pH 6.0.

Substrate	nmol min^{-1} (mg prot.)$^{-1}$	
	Potato leaf	Potato tuber
PNP-Phosphorylcholine	4.6	8.5
PC	31.3	2.2
CDP-choline	5.5	3.8
Glycerol phosphoryl-choline	3.6	6.7

Table III. Partial purification of the PNP-PC hydrolase activity from potato leaves.

Step	Protein (mg)	Total act. (nmol min^{-1})	Specific act. (nmol min^{-1} (mg prot.)$^{-1}$)	% Recovery	Purification (fold)
100 000 × *g* Supernatant	351	1369	3.9	100	—
Sephadex G-25	202	1359	8.04	99.3	2.06
DEAE-cellulose	49.2	1195	24.3	87.3	6.23
Red-A	19.7	1098	55.7	80.2	14.3
AcA-34	1.37	163	118.8	11.9	30.5
Chromatofocus (peak at pH 6.8)	0.057	51	894.6	3.7	229

void volume was then incubated at 4°C overnight and a substantial amount of white precipitate was consistently found and was removed by centrifugation at 10 000 × *g* for 30 min. The clarified enzyme sample contained all of the original enzyme activity and was then applied to a DEAE cellulose column. Although most of the enzyme activity passed through the DEAE column without binding, about 75% of the other proteins did bind, thus providing about a 3-fold purification. All of enzyme activity was bound to the Red-A column and was eluted during an early stage of the KCl gradient (Fig. 1A). The Red-A column gave a 2.3-fold purification. The gel filtration step gave a further purification of about 2-fold (Fig. 1B). The final step of purification consisted of a chromatofocusing column. The enzyme was eluted at pH 6.8 and a further purification of 7.5-fold was achieved (Fig. 1C). Using this purification scheme we achieved a 229-fold purification of the PNP-PC hydrolase and a 3.7% recovery. When potato tubers were homogenized and subjected to the same protocol, a 66-fold purification was achieved and the properties of the tuber enzyme activity appeared to be identical to those of the leaf enzyme activity (data not shown). When the partially purified enzyme preparations from leaves and tubers were analyzed by SDS-PAGE, 4—6 proteins bands were detected. This indicated that although a significant degree of purification had been achieved, the enzyme preparations were not homogeneous.

Properties of the PNP-PC hydrolase

The molecular weight of the partially purified enzyme was estimated to be about, 28 000 by gel filtration chromatography (Fig 2). Because the enzyme eluted from the chromatofocusing column (Fig. 1C) at pH 6.8, we estimate that the P_i is approximately equal to this pH value or perhaps is slightly higher. When the activity of the partially-purified PNP-PC hydrolase was measured at several pH values from 4 to 9, a broad peak of activity was obtained. with the highest activity at pH 6.0 (Fig. 3). The activity of the partially-purified enzyme was also measured with various concentrations of substrate, ranging from 0.2 to 20 mM PNP-PC (Fig. 4). When these data were plotted on a Lineweaver-Burk plot the K_m of the enzyme for PNP-PC was found to be very high, approx. 10 mM. Two common divalent cations (Ca^{2+} and Zn^{2+}) and ethylene diaminetetraacetic acid (EDTA) were then tested to determine their effect on the partially purified enzyme preparation (Fig. 5). A significant inhibition was obtained with Zn^{2+} at concentrations of 1—5 mM. In contrast, Ca^{2+} and EDTA each yielded only a slight inhibition in the range of concentrations studied.

Ability of partially purified hydrolase to hydrolyze various substrates

The substrate specificity of the partially purified enzyme was then determined (Table IV). Although PNP-PC has been suggested to be a convenient substrate for phospholipase C [7], the inability of this enzyme preparation to hydrolyze PC indicated the absence of phospholipase C activity. In addition, two other common phospholipids, phospha-

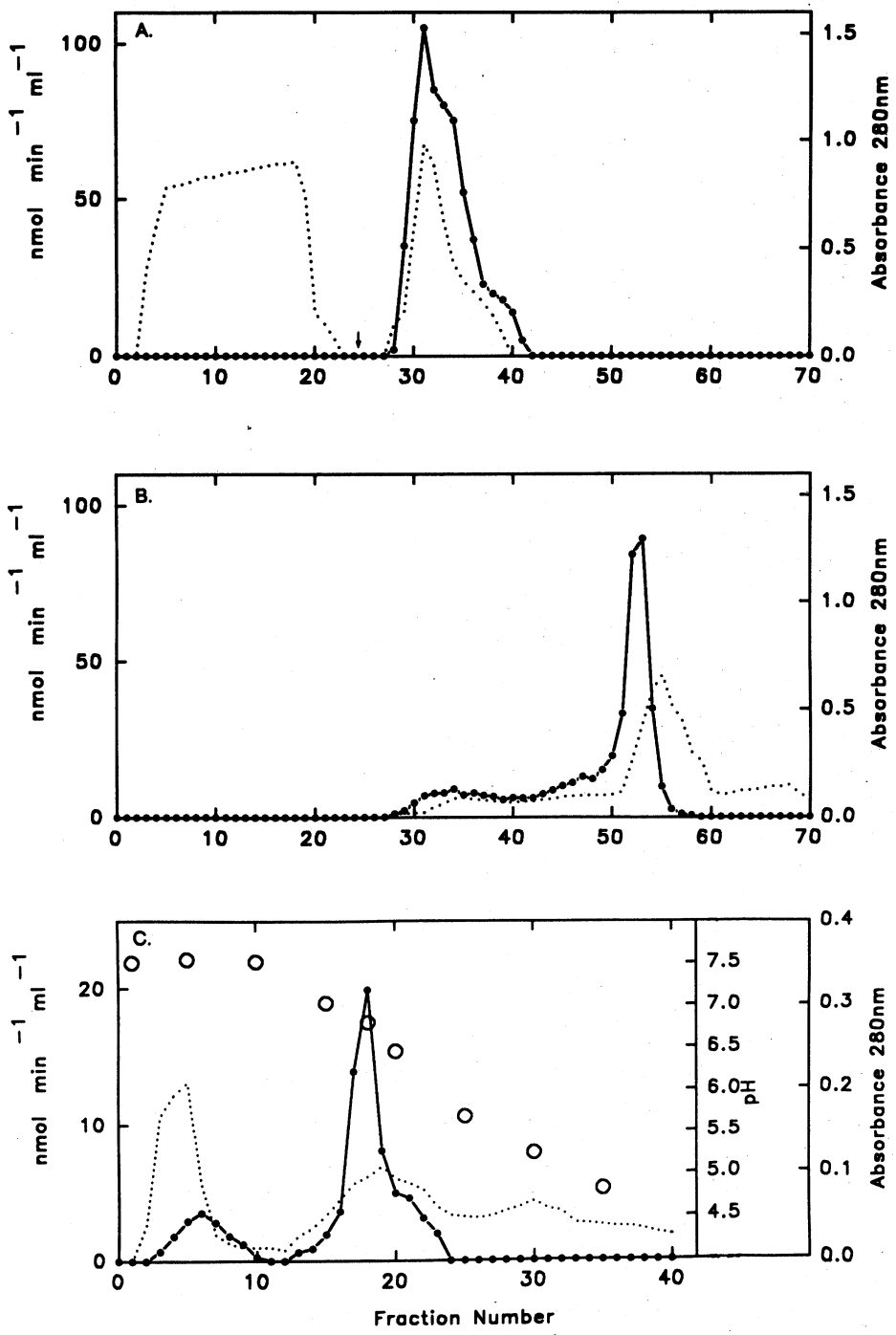

Fig. 1. Major steps in the purification of PNP-PC hydrolase. (A) Dye matrix chromatography on a Red A (Reactive Red 120-Agarose) column. At the arrow a linear gradient (0—0.1 M) of KCl was started. (B) Gel filtration chromatography on LKB Ultrogel AcA-34. (C) Chromatofocusing with Pharmacia Polybuffer Exchanger 94 equilibrated at pH 7.4 and eluted with Pharmacia Polybuffer 74 adjusted to pH 4.0. ●, enzyme activity (rate of hydrolysis of PNP-PC); ○, pH of chromatofocus fractions; ....., absorbance at 280 nm.

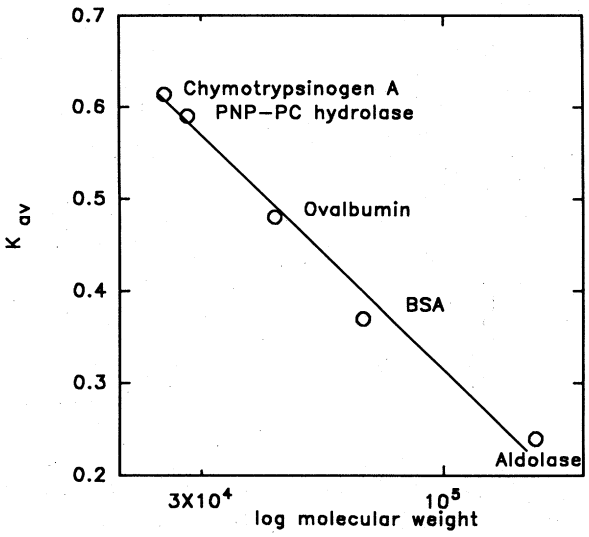

Fig. 2. Estimation of the molecular weight of PNP-PC hydrolase by gel filtration chromatography on LKB Ultrogel AcA-34 with protein standards of known molecular weight.

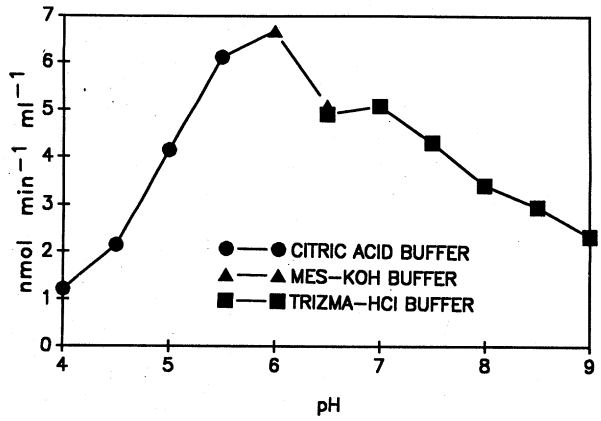

Fig. 3. The effect of pH on the activity of partially purified PNP-PC hydrolase from potato leaves. Each buffer was tested at a concentration of 50 mM. ●, citric acid buffer; ▲, Mes-KOH buffer; □, Trizma—HCl buffer.

tidylethanolamine and phosphatidylinositol, were also tested and found not to serve as substrates for the partially purified enzyme. When two other phosphorylcholine-containing metabolites were then tested for activity, CDP-choline was found to be hydrolyzed at a rate similar to the rate of hydrolysis of PNP-PC and glycerol phosphorylcholine was not hydrolyzed. This experiment indicates that the PNP-PC hydrolase may serve as a CDP-choline hydrolase in vivo, but not as a phospholipase C. The experiment also indicates that although the leaves and

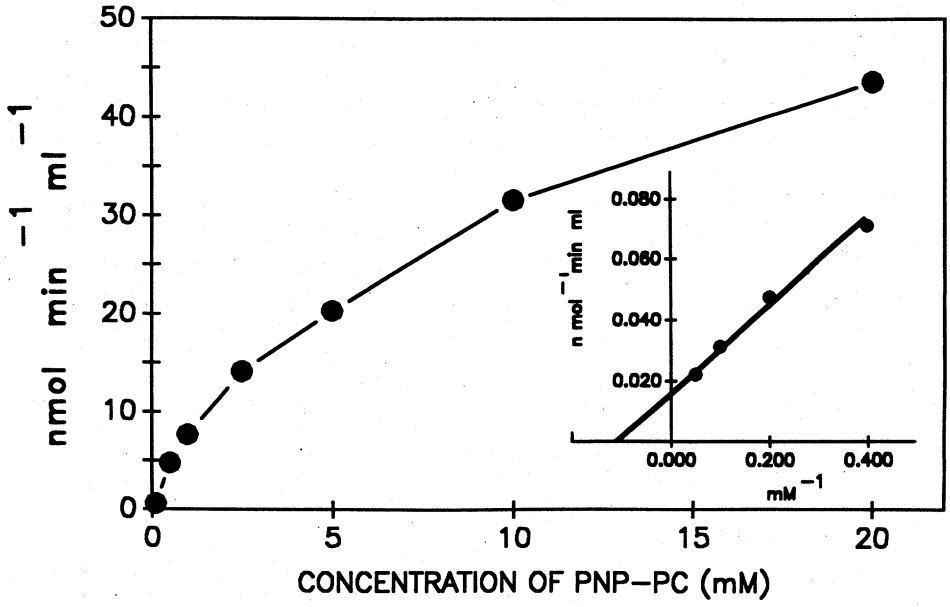

Fig. 4. The effect of concentration of substrate (PNP-PC) on the activity of PNP-PC hydrolase.

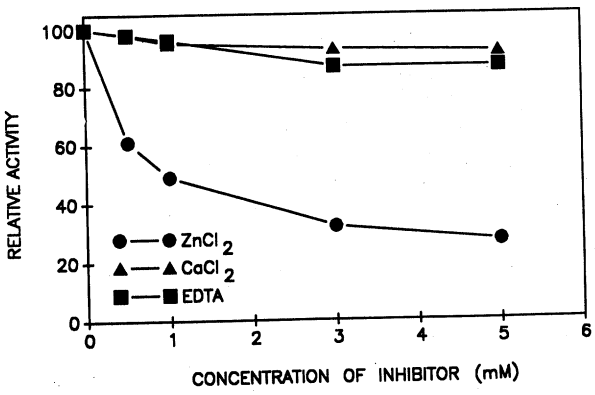

Fig. 5. Effect of various concentrations of Zn^{2+}, Ca^{2+} and EDTA on the activity of PNP-PC hydrolase. ●, $ZnCl_2$; ▲, $CaCl_2$; □, EDTA.

tubers of *S. tuberosum* are capable of hydrolyzing all three phosphorylcholine-containing metabolites (Table II), the enzyme we have purified can only hydrolyze CDP-choline (and the artificial substrate, PNP-PC).

Discussion

Although PNP-PC has been recommended as a suitable artificial substrate for phospholipase C-type enzymes [7] this study reveals that other types of plant enzymes are able to hydrolyze PNP-PC.

Table IV. Comparison of the rates of hydrolysis of various phosphorylcholine-containing metabolites by partially purified PNP-PC hydrolase from potato leaves. All assays were buffered at pH 6.0

Substrate	Potato leaf hydrolase activity
	nmol min^{-1} (mg prot.)$^{-1}$
PNP-Phosphorylcholine	755
PC	0
CDP-choline	784
Glycerol phosphoryl-choline	0

Although our initial studies indicated that potato leaves contained a phospholipase C activity [11], when the PNP-PC hydrolase from potato leaves was further purified in this study, the only physiological substrate that was hydrolyzed by the enzyme was CDP-choline.

This is the first report of plant tissues which contain enzymes that are capable of hydrolyzing either CDP-choline or glycerol phosphorylcholine (Table II). Since potato tubers and leaves each contain high levels of lipolytic acyl hydrolase (LAH), it is not surprising that they also contain an enzyme which is capable of degrading glycerol phosphorylcholine (which originates from the action of LAH on PC).

The hydrolysis of each of the four phosphorylcholine-containing compounds used in this study yielded phosphorycholine as a common product. Very little is known about the fate of phosphorylcholine in plant tissues. There may be other hydrolases which can degrade phosphorylcholine. We can only state that in our studies of the hydrolysis of CDP-choline and glycerol phosphorylcholine, phosphorylcholine accumulated at concentrations sufficient for us to accurately measure its rate of appearance.

CDP-choline is thought to participate in the synthesis of PC in some plants via the enzyme CDP-choline diacylglycerol phosphotransferase [12]. Since the CDP-choline DAG phosphotransferase is thought to be located in the mitochondria and endoplasmic reticulum [11], it may be spatially separated from the soluble CDP-choline hydrolase which we have identified in the present work. From our current state of knowledge it is difficult to speculate on the physiological role of a CDP-choline hydrolase. However, since plants can actually synthesize PC by a second pathway (which involves the methylation of phosphatidyl ethanolamine) [12] it is possible that under certain conditions (i.e. when DAG is needed for other metabolic purposes) the CDP-choline hydrolase may function in shutting down the CDP-choline DAG phosphotransferase pathway.

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